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Lactosaminated and intact *N*-succinyl-chitosans as drug carriers in liver metastasis

Yoshinori Kato *, Hiraku Onishi, Yoshiharu Machida

Department of Drug Delivery Research, Hoshi University, 2-4-41 Ebara, Shinagawa-ku, Tokyo 142-8501, Japan

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Abstract

The biodistributions of fluorescently labeled *N*-succinyl-chitosan (Suc-FTC) and lactosaminated *N*-succinyl-chitosan (Lac-Suc-FTC) after i.v. administration to mice intravenously inoculated with M5076 cells were investigated at 3 and 12 days post-inoculation. At both time points, Lac-Suc-FTC was specifically localized to the liver. However, the area under the concentration–time curve in the liver decreased gradually by progress of the liver metastasis. At 3 days post-inoculation, Suc-FTC showed good retention in the systemic circulation and was little distributed to the liver. However, at 12 days post-inoculation, Suc-FTC was eliminated relatively fast from the systemic circulation and gradually accumulated in the liver. The antitumor effects of mitomycin C (MMC), Lac-Suc-MMC conjugate (Lac-Suc-MMC) and highly succinylated Suc (Suc(II))-MMC conjugate (Suc(II)-MMC) were examined on single i.v. administration for both metastatic stages. For administration at 3 days post-inoculation, Lac-Suc-MMC alone tended to elongate significantly the lifespan at a lower dose (0.4 mg eq. MMC/kg), and MMC, Suc(II)-MMC and Lac-Suc-MMC increased significantly the lifespan at a higher dose (10 mg eq. MMC/kg). However, at 12 days post-inoculation (late stage of metastasis), neither MMC nor the conjugates were effective even at the higher dose (10 mg eq. MMC/kg). Both carriers, Suc showing systemic long-circulation and Lac-Suc with an ability of liver-specific localization, are thought to be drug carriers with potentialities for therapeutics at early stage of metastasis.

Keywords: *N*-Succinyl-chitosan; Lactosaminated *N*-succinyl-chitosan; Mitomycin C; M5076 liver metastasis; Biodisposition; Antitumor effect

1. Introduction

In cancer chemotherapy, it is important that the antitumor drugs are delivered to the tumor sites efficiently in order to reduce the severity of side effects. Many systems have been used to deliver drugs to the target sites (Gregoriadis, 1977; Maeda, 1991). Above all, we have focused on *N*-succinyl-chitosan (Suc) as a drug carrier for active and passive targeting. Suc has excellent characteristics as a drug carrier, i.e. long-term retention in the systemic circulation (Kamiyama

* Corresponding author. Tel.: +81-3-5498-5760; fax: +81-3-5498-5759.

E-mail address: d711@hoshi.ac.jp (Y. Kato).

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et al., 1999; Kato et al., 2000a), low toxicity (Song et al., 1993) and good drug loading (Song et al., 1992). In fact, when a water-soluble macromolecular prodrug of mitomycin C with highly succinylated *N*-succinyl-chitosan (Suc(II)), Suc(II)-MMC, was injected into Sarcoma 180-bearing mice, it was accumulated in Sarcoma 180 solid tumor due to the enhanced permeability and retention (EPR) effects, and good antitumor activity was found with reduced side effects (Kato et al., 2000b). Furthermore, it was demonstrated in normal mice that the introduction of lactose to Suc enabled liver targeting of the carrier (Kato et al., 2001). However, it is known to be difficult for the macromolecules with galactose moieties to target to the liver effectively under diseased conditions (Virgolini et al., 1990; Pimm et al., 1996). It is also known that the angioarchitecture of hepatocellular carcinoma changes markedly as the cancer progresses (Kojiro, 2000; Kudo, 2000). Therefore, the biodistribution of macromolecules with galactose residues is considered to be affected by liver metastasis. In addition, it is unknown how Suc possessing no lactose behaves in mice with liver metastasis. Thus, in the present study, the biodistribution of Lac-Suc and Suc has been examined using mice bearing experimental liver metastasis of M5076 cells (Perez-Soler et al., 1987; Yachi et al., 1996) in the early and advanced stage of the disease. Further, conjugates of mitomycin C were investigated in vivo for antitumor characteristics using M5076-bearing mice.

2. Materials and methods

2.1. Materials

Mitomycin C (MMC) was purchased from Kyowa Hakko Kogyo Co (Tokyo, Japan). MMC was used after extraction by methanol. *N*-Succinyl-chitosan sodium salt (Suc: succinylation degree 0.81 mol per sugar unit, deacetylation degree 1.0 mol per sugar unit, MW 3.4×10^5) was kindly supplied by Katakura Chikkarin Co Ltd (Tokyo, Japan). Lactose and fluorescein isothiocyanate (FITC) were purchased from Sigma Chemical Company (St. Louis, USA). Sodium cyanoborohydride was purchased from Tokyo Kasei Kogyo Co Ltd (Tokyo, Japan). All other chemicals were obtained commercially as reagent-grade products.

2.2. Animals and tumors

Specific-pathogen-free male mice of the inbred strain C57BL/6 weighing approximately 20 g (at the age of 6 weeks) were purchased from Tokyo Laboratory Animals Science Co Ltd (Tokyo, Japan) and used soon after for the experiments. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Hoshi University. Four animals were used for each time point in distribution experiments on Lac-Suc-MMC and Suc(II)-MMC ($n = 4$), and five animals in each group for in vivo antitumor tests ($n = 5$). For all other experiments, three animals were used ($n = 3$).

Murine reticulum cell sarcoma M5076 cells were used as tumor cells. These cells metastasize to several organs including the liver. M5076 cells were maintained in C57BL/6 mice by intraperitoneal transfer of 1×10^5 cells obtained from ascitic fluid every other week. In the in vivo biodistribution experiments and antitumor activity tests, 1×10^5 M5076 cells suspended in 0.1 ml of Hanks' balanced salt solution, which were obtained from the above mice bearing the tumor intraperitoneally, were inoculated intravenously into each mouse.

2.3. Biodistribution studies of lactosaminated *N*-succinyl-chitosan and *N*-succinyl-chitosan

Lactosaminated Suc (Lac-Suc) was synthesized by reductive amination using sodium cyanoborohydride according to a method reported earlier (Kato et al., 2001). The lactose residue content of Lac-Suc was determined to be about 0.30 mol per sugar unit by elemental analysis (Yanako Analytical Industrial Co, Japan).

Fluorescein isothiocyanate-labeled Lac-Suc (fluorescein thiocarbamyl (FTC) Lac-Suc), abbreviated as Lac-Suc-FTC, and fluorescein isothiocyanate-labeled Suc, abbreviated as Suc-FTC, were used throughout the biodistribution experiments. Lac-Suc-FTC and Suc-FTC were prepared

as reported earlier (Kato et al., 2000a). The FTC contents of Lac-Suc-FTC and Suc-FTC, examined according to the earlier report (Kato et al., 2000a), were 1.5 and 2.3% (w/w), respectively.

The recoveries of Suc-FTC and Lac-Suc-FTC from various tissues were determined using normal mice according to the method of Kamiyama et al. (1999).

To investigate the biodistribution of Lac-Suc-FTC administered at an early stage of liver metastasis, at 3 days after i.v. tumor inoculation, M5076-bearing mice received Lac-Suc-FTC via the lateral tail vein at a dose of 0.2 mg (0.2 ml) per mouse without anesthetic. The mice were sacrificed at 5 min, 0.5, 1, 8, 24 and 48 h after injection, blood samples were withdrawn and several tissues (liver, kidney, spleen and lung) were excised. The subsequent procedure was performed as reported earlier (Kato et al., 2000a). The concentration was corrected by the recoveries. The distributed amount was calculated from the concentration and tissue weight. The corrected plasma concentration means the concentration given provided Lac-Suc-FTC was completely transferred from blood to plasma. The amount of Lac-Suc-FTC in plasma was calculated using the reported volume of mouse plasma, 48.8 ml/kg (Tajima, 1989).

To investigate the biodistribution of Lac-Suc-FTC when administered at an advanced stage of liver metastasis, at 12 days after i.v. tumor inoculation, M5076-bearing mice received Lac-Suc-FTC via the lateral tail vein at a dose of 0.2 mg (0.2 ml) per mouse with no anesthetic. The subsequent procedures were the same as those described for the early stage of liver metastasis.

The biodistribution of Suc-FTC was examined in the same way as that of Lac-Suc-FTC except that the body distribution at 5 min and 0.5 h after i.v. injection was not investigated.

2.4. Pharmacokinetic analysis

The areas under the plasma or liver concentration–time curves for 0–48 h ($AUC_{0-48\ h}$) and the mean residence time ($MRT_{0-48\ h}$) were calculated by the trapezoidal method (Yamaoka et al., 1981). The following equation was employed to determine the relative effectiveness of liver targeting (R_{et}):

$$R_{et} = \frac{AUC^{liver}_{0-48\ h}}{AUC^{plasma}_{0-48\ h}}$$

2.5. Urinary excretion

The cumulative collection of urine was performed simultaneously with the biodistribution studies as described above. Mice were placed separately in metabolite cages immediately after administration. Urine was collected for 8, 24 and 48 h after i.v. administration, and then urinary volume was measured. Each urine sample was filtered using a membrane filter (0.45 μm pore diameter). The filtrate was diluted appropriately with phosphate buffered saline (PBS), and fluorescence intensities were measured fluorometrically (Ex. 495 nm, Em. 520 nm). The total amount excreted in urine was calculated from the concentration and urinary volume.

2.6. In vivo antitumor effects of Suc(II)-MMC and Lac-Suc-MMC conjugates against M5076 liver metastatic tumor

First, in order to give a water-soluble conjugate, highly-succinylated Suc (Suc(II)) was prepared by reaction of Suc with succinic anhydride as reported earlier (Kato et al., 2000b). Namely, although most of the conjugate prepared by the direct coupling of MMC with original Suc using water-soluble carbodiimide was water-insoluble due to crosslinking among or within the polymer supports, water-soluble conjugate could be obtained to a much larger extent by using Suc(II) instead of Suc (Kato et al., 2000b). The preparation of the water-soluble Suc(II)-MMC conjugate (Suc(II)-MMC) followed the earlier manner (Kato et al., 2000b). Lac-Suc-MMC conjugate (Lac-Suc-MMC) was also prepared by the same procedure except that Lac-Suc was used instead of Suc(II). Suc(II)-MMC and Lac-Suc-MMC showed the drug contents of 12 and 20% (w/w), respectively, which were measured in the manner reported earlier (Kato et al., 2000b). These were used throughout the study. The dose of MMC conju-

gates was expressed in terms of amount of parent MMC.

Antitumor effects were examined using the mice at 3 and 12 days after intravenous inoculation with M5076 cells. Namely, at 3 or 12 days post-inoculation, MMC was intravenously administered at a single dose of 0.4, 5 and 10 mg/kg, and Suc(II)-MMC or Lac-Suc-MMC were intravenously administered at a single dose of 0.4 and 10 mg eq. MMC/kg, respectively. Controls were injected with a similar volume of saline alone. For all the mice, the survival time after inoculation was observed for 50 days post-inoculation. The antitumor effects were obtained by comparing the mean survival time of the treated mice after inoculation (T) with that of the control mice after inoculation (C), that is, from the increase in life span (ILS) calculated by the following equation:

$$\mathrm{ILS} = \left(\frac{T}{C} - 1\right) \times 100(\%)$$

At the same time, the changes in body weight of each group were measured to evaluate the toxic side effects.

2.7. Distribution of free and conjugated MMCs in blood circulation and liver after i.v. injection of the conjugates

In this experiment, the mice were used at 3 and 12 days post-inoculation. Lac-SucMMC or Suc(II)-MMC (4 mg eq. MMC/kg) was intravenously injected into M5076-bearing mice. The mice were sacrificed at 8 and 24 h after injection, the liver was enucleated, and plasma was obtained. The same volume of 1 M sodium carbonate buffer (pH 9.0) was added to the excised liver, and the mixture was homogenized with a glass homogenizer with a Teflon pestle.

Free MMC contained in the obtained sample mixture (pH 9.0) was extracted according to the method of Den Hartigh et al. (1981). This operation allowed the complete recovery of MMC from the sample. The extracted MMC was finally dissolved in methanol. The amount of free MMC in the sample was determined by high performance liquid chromatography (HPLC).

To determine the total amounts of free and conjugated MMC in the obtained sample mixture (pH 9.0), heating treatment (90 °C, 5 min) was performed (Kato et al., 2000b,c); this operation allowed the almost complete release of MMC from the conjugates. Then, MMC was extracted by the same method, and finally dissolved in methanol. The total amounts of free and conjugated MMC in the sample were determined by HPLC.

HPLC was executed at room temperature, and the absolute calibration curve method was used for analysis (Den Hartigh et al., 1981). Samples (20 μl of the final solution in methanol) were injected into a SUMIPAX Nucleosil $5C_{18}$ reversed phase column (4 mm in inner diameter × 250 mm in length) and eluted at a flow rate of 0.4 ml/min with a mobile phase of a mixture of 0.01 M phosphate buffer, pH 6.0, and methanol (65:35, v/v). The HPLC operation was performed using a Shimadzu LC-6AD apparatus equipped with an SPD-10AV UV detector (Shimadzu) set at 365 nm. The concentration of MMC was calculated using a standard curve.

2.8. Statistical analysis

Student's t-test was performed to determine the level of significance without survival analysis. As to survival, the Mantel–Cox log-rank test was applied to check for a significant difference. The data were considered to be significantly different when the P-value was less than 0.05.

3. Results

3.1. Body distribution of Lac-Suc-FTC and Suc-FTC

The concentrations of Lac-Suc-FTC distributed after i.v. administration at a dose of 0.2 mg per M5076-bearing mouse at 3 and 12 days after i.v. tumor inoculation are shown in Fig. 1(A) and (B), respectively. Lac-Suc-FTC was quickly accumulated in the liver and showed very little accumulation in other tissues. Furthermore, only less than 2.5% of the dose administered remained in plasma

at 8 h after injection. At 1 h post-injection, the amount in liver was more than 15-fold that in any other tissue except plasma (Fig. 1(A-2)). Even at 12 days post-inoculation, Lac-Suc-FTC was rapidly accumulated in the liver and showed little distribution to other tissues except the kidney at 1 h (Fig. 1(B)); however, the liver concentration at 12 days post-inoculation was lower than that at 3 days post-inoculation. Furthermore, less than 2.3% of the dose remained in plasma at 8 h after injection.

Fig. 2(A) and (B) also show the concentrations of Suc-FTC distributed after i.v. administration at a dose of 0.2 mg per M5076-bearing mouse at 3 and 12 days post-inoculation, respectively. In contrast to Lac-Suc-FTC, at 3 days post-inoculation, Suc-FTC was retained in the blood circulation at a high level for at least 48 h and was slightly distributed to the liver (Fig. 2(A-1)). On the other hand, at 12 days post-inoculation, Suc-FTC was eliminated more rapidly from the systemic circulation (Fig. 2(B-1)). The amount distributed in the liver was greater at 12 days than at 3 days post-inoculation. The localization to the spleen was also increased at 12 days post-inoculation.

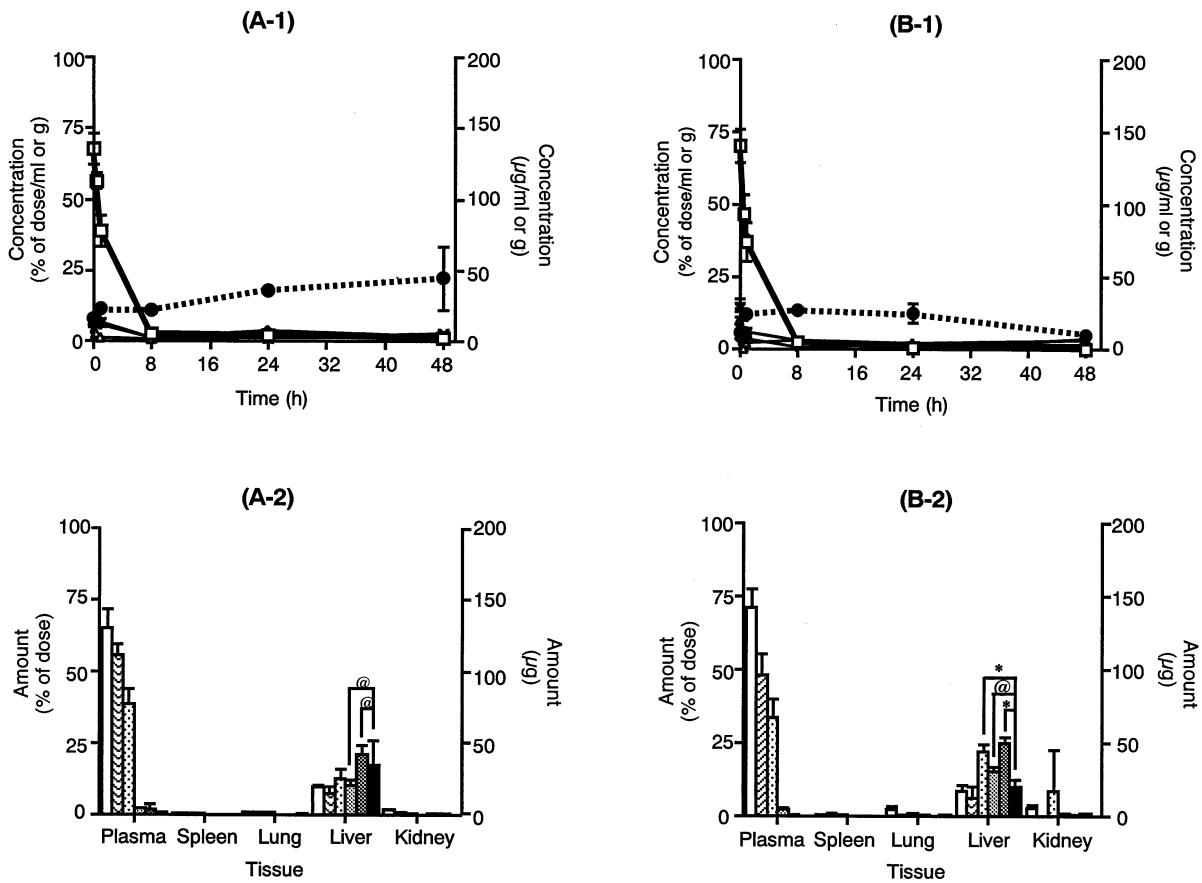

Fig. 1. Plasma concentration and tissue distribution of Lac-Suc-FTC after i.v. administration at a dose of 0.2 mg (0.2 ml) per M5076-bearing mouse. —□—, Plasma, ; Liver, —△—; Kidney, ; Lung, —◇—; Spleen. At 3 days ((A-1), (A-2)) or 12 days ((B-1), (B-2)) after i.v. inoculation, test substance (0.2 ml) was injected intravenously. Each point represents the mean ± S.D. ($n = 3$). (A-2) 3 days (□: 5 min, : 0.5 h, : 1 h, : 8 h, : 24 h, ■: 48 h). (B-2) 12 days (□: 5 min, : 0.5 h, : 1 h, : 8 h, : 24 h, ■: 48 h).

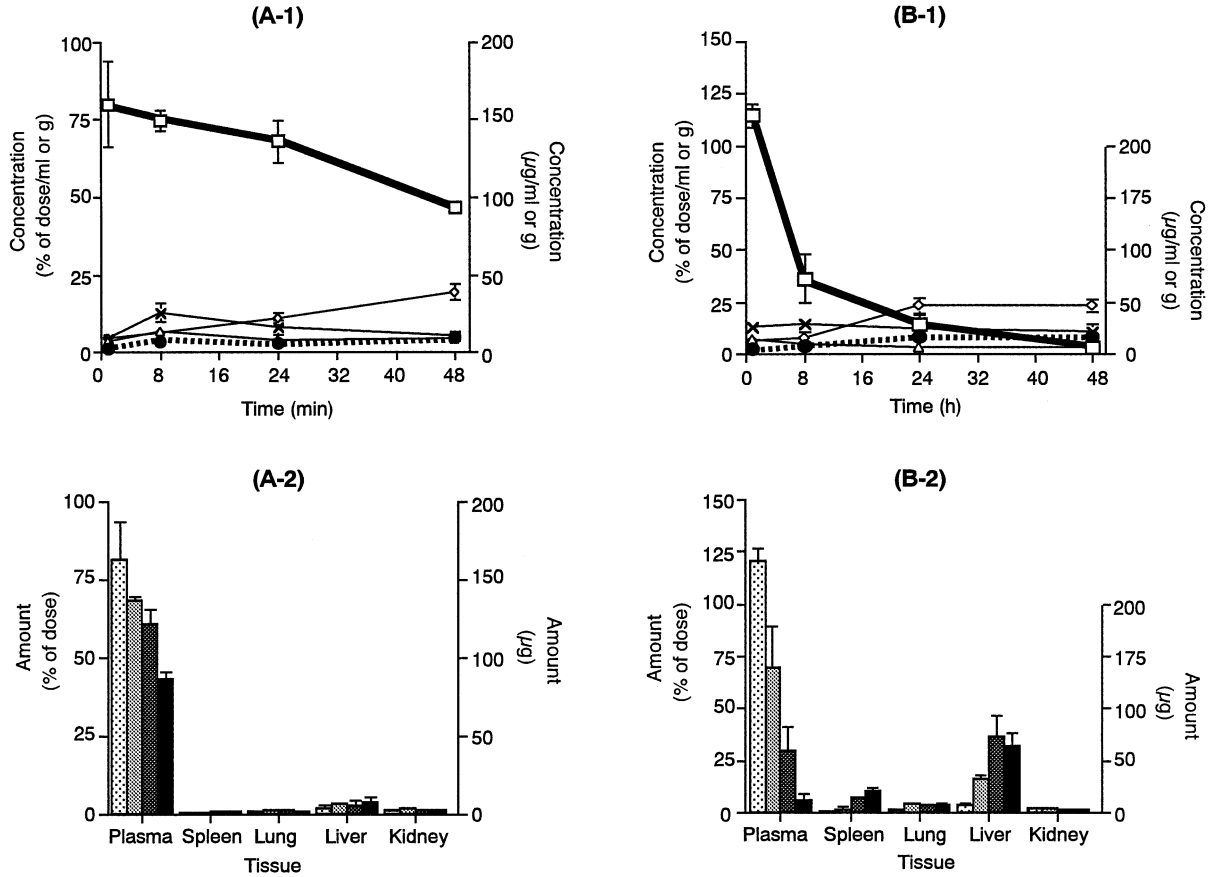

Fig. 2. Plasma concentration and tissue distribution of Suc-FTC after i.v. administration at a dose of 0.2 mg (0.2 ml) per M5076-bearing mouse. —□—; Plasma, ····●···· ; Liver, —△—; Kidney, —×— ; Lung, —◇—; Spleen. At 3 days ((A-1), (A-2)) or 12 days ((B-1), (B-2)) after i.v. inoculation, test substance (0.2 ml) was injected intravenously. Each point represents the mean ± S.D. ($n = 3$). (A-2) 3 days (▨: 1 h, ▨: 8 h, ▨: 24 h, ■: 48 h). (B-2) 12 days (▨: 1 h, ▨: 8 h, ▨: 24 h, ■: 48 h).

3.2. Pharmacokinetics of Lac-Suc-FTC and Suc-FTC

The pharmacokinetic parameters of Lac-Suc-FTC at a dose of 0.2 mg per mouse, estimated from the mean concentration–time curves in the plasma and liver, are shown in Table 1(A). The data at 0 days, corresponding to the data for normal mice, are obtained from the earlier results using normal mice (Kato et al., 2001). AUC, MRT and R_{et} calculated based on the concentration–time curve, were abbreviated to AUC(C), MRT(C) and $R(C)_{et}$, respectively. $AUC(C)_{0-48\ h}^{liver}$ at 12 days post-inoculation was two-thirds of that at 3 days post-injection. Moreover, $AUC(C)_{0-48\ h}^{plasma}$ at 12 days post-inoculation was four-fifths of that at 3 days post-injection. After all, the $R(C)_{et}$ value at 3 days post-inoculation was greater than that at 12 days post-injection. The $MRT(C)_{0-48\ h}$ values in the plasma and liver were larger at 3 days post-inoculation. Both $AUC(C)_{0-48\ h}$ and $R(C)_{et}$ values decreased gradually with progression of liver metastasis.

The pharmacokinetic parameters of Lac-Suc-FTC at a dose of 0.2 mg per mouse, estimated from the mean amount–time curves in the plasma and liver, are shown in Table 1(B). AUC, MRT and R_{et} calculated and evaluated based on the amount–time curve, were abbreviated as AUC(M), MRT(M) and $R(M)_{et}$, respectively. The AUC

Table 1
Pharmacokinetic parameters of Lac-Suc-FTC after i.v. administration at a dose of 0.2 mg (0.2 ml) per M5076-bearing mouse

Injection day post-inoculation (days)		(A) concentration			(B) amount		
		$AUC(C)_{0-48\ h}$ (h.μg/ml or g)	$MRT(C)_{0-48\ h}$ (h)	$R(C)_{et}$[a]	$AUC(M)_{0-48\ h}$ (h.μg mouse)	$MRT(M)_{0-48\ h}$ (h)	$R(M)_{et}$[a]
0[b]	Liver	1860	19	3.4	1580	20	3.3
	Plasma	550	1.5		480	1.5	
3	Liver	1610	28	2.9	1620	26	3.1
	Plasma	550	7.0		530	6.8	
12	Liver	1020	20	2.4	1770	22	4.3
	Plasma	430	2.7		410	2.8	

[a] R_{et} means the relative effectiveness of liver targeting, and was calculated using the following equation:

$$R_{et} = \frac{AUC^{liver}_{0-48\ h}}{AUC^{plasma}_{0-48\ h}}.$$

[b] The data in 0 days were from the earlier results of Kato et al. (2001) using normal mice.

$(M)^{liver}_{0-48\ h}$ values slightly increased with the progression of liver metastasis. The $AUC(M)^{plasma}_{0-48\ h}$ value was a little lower at 12 days than at 3 days post-inoculation. Accordingly, the $R(M)_{et}$ value for Lac-Suc-FTC increased slightly with the progression of liver metastasis.

Therefore, when liver metastasis was progressed, the concentration data indicated small decrease in ability of Lac-Suc-FTC for liver targeting, but the amount localized to the liver was rather raised to some extent. This increase in the amount localized to the liver was found to be due to liver size, which was increased with progression of liver metastasis.

$AUC^{liver}_{0-48\ h}$, AUC^{plasma}_{0-48h}, $MRT^{liver}_{0-48\ h}$, $MRT^{plasma}_{0-48\ h}$ and R_{et} values of Suc-FTC at 3 and 12 days post-inoculation were calculated as shown in Table 2. In the advanced stage of liver metastasis at 12 days post-inoculation, the $AUC(C)^{plasma}_{0-48\ h}$ and $AUC(M)^{plasma}_{0-48\ h}$ were about two-fifths of those at 3 days post-injection, respectively. On the other hand, at the advanced stage of liver metastasis at 12 days post-inoculation, the $AUC(C)^{liver}_{0-48\ h}$ and $AUC(M)^{liver}_{0-48\ h}$ were increased by 2- and 4.7-fold the values at 3 days post-inoculation, respectively. As shown in Table 2, the $R(C)_{et}$ and $R(M)_{et}$ values at 12 days post-inoculation were 5 and 10 times larger than those at 3 days post-injection, respectively. Therefore, the liver localization of Suc-FTC increased as to both concentration and amount.

3.3. Urinary excretion

The levels of urinary excretion of Lac-Suc-FTC and Suc-FTC are shown in Fig. 3. The urine collected in the cage bottle and that withdrawn from the bladder were combined to obtain the total urinary excretion. As shown in Fig. 3(A), the amounts of Lac-Suc-FTC excreted were not different between the early and advanced stages of liver metastasis. The urinary excretion of Suc-FTC tended to be lower at 12 days post-inoculation than at 3 days post-inoculation (Fig. 3(B)), but it was not significantly different between the two stages ($P > 0.05$).

3.4. In vivo antitumor effect of Suc(II)-MMC and Lac-Suc-MMC against M5076-bearing mice

Fig. 4(A) illustrates the ratio of survival and the change of body weight when MMC, Suc(II)-MMC or Lac-Suc-MMC was administered at 3 days post-inoculation. Lac-Suc-MMC at a dose of 10 mg eq. MMC/kg was observed to produce the highest ILS value, i.e. 91.3%. The survival on administration of MMC (5 mg/kg), Suc(II)-MMC (10 mg eq. MMC/kg) and Lac-Suc-MMC (10 mg eq. MMC/kg) was significantly different from that of control. However, a significant difference in survival was not found among MMC (5 mg/kg),

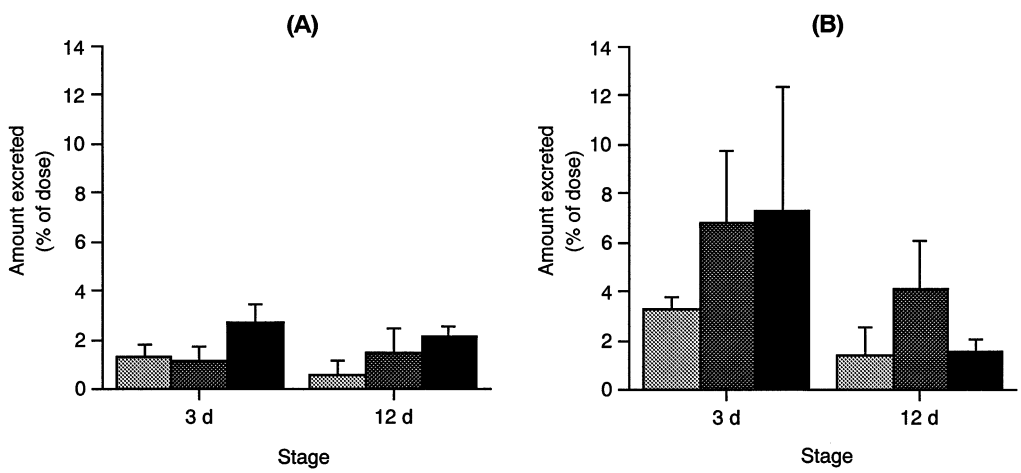

Fig. 3. Urinary excretion of Lac-Suc-FTC and Suc-FTC after i.v. administration at a dose of 0.2 mg (0.2 ml) per M5076-bearing mouse. (A) Lac-Suc-FTC (▩: 8 h, ▨: 24 h, ■: 48 h). (B) Suc-FTC (▩: 8 h, ▨: 24 h, ■: 48 h). Each column represents the mean ± S.D. ($n = 3$).

Table 2
Pharmacokinetic parameters of Suc-FTC after i.v. administration at a dose of 0.2 mg (0.2 ml) per M5076-bearing mouse

Injection day post-inoculation (days)		(A) concentration			(B) amount		
		$AUC(C)_{0-48\ h}$ (h.μg/ml or g)	$MRT(C)_{0-48\ h}$ (h)	$R(M)_{et}$[a]	$AUC(C)_{0-48\ h}$ (h.μg per mouse)	$MRT(M)_{0-48\ h}$ (h)	$R(M)_{et}$[a]
3	Liver	320	28	0.05	280	26	0.05
	Plasma	6270	21		5770	21	
12	Liver	640	29	0.25	1340	28	0.52
	Plasma	2530	9.9		2600	9.9	

[a] R_{et} means the relative effectiveness of liver targeting, and was calculated using the following equation:

$$R_{et} = \frac{AUC^{liver}_{0-48\ h}}{AUC^{plasma}_{0-48\ h}}.$$

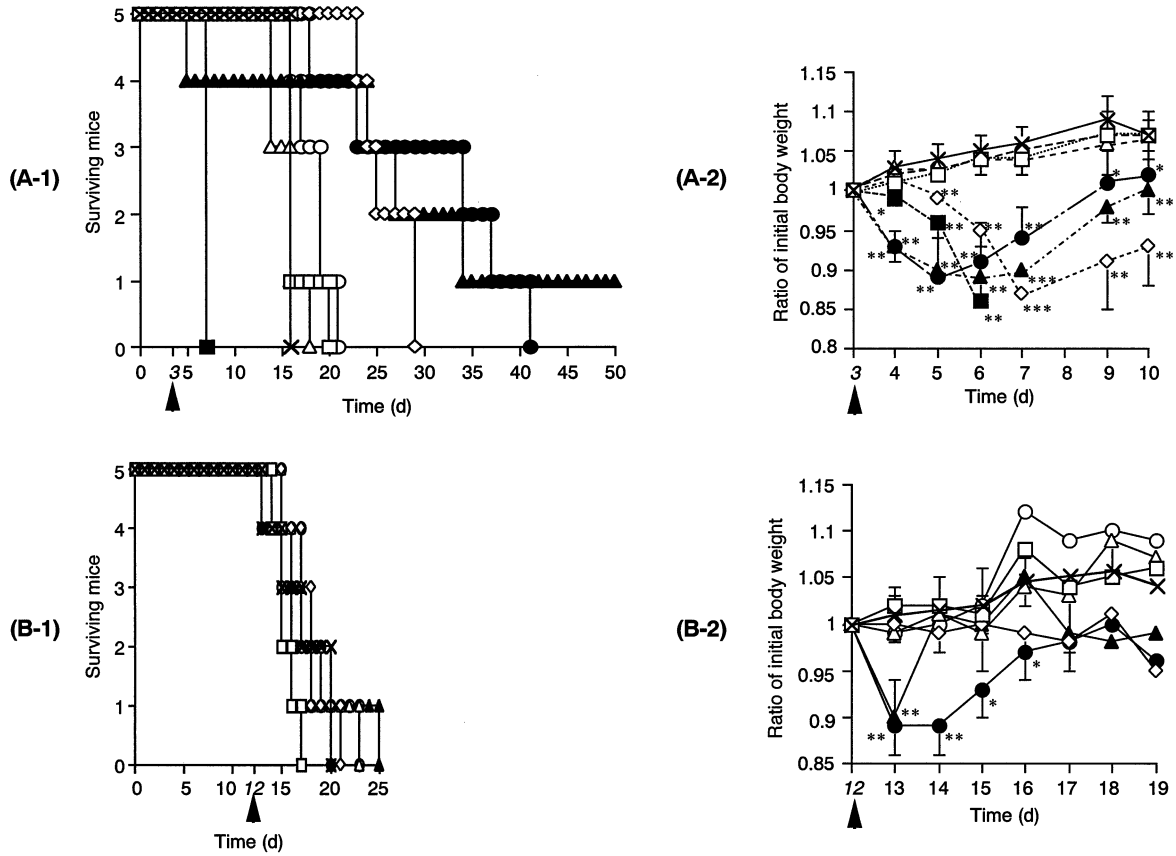

Fig. 4. Effect of MMC, Suc(II)-MMC and Lac-Suc-MMC on the survival of M5076-bearing mice (A-1, B-1) and change in the mean body weight of mice bearing M5076 (A-2, B-2) after i.v. administration at 3 or 12 days post-inoculation. At 3 days (▲) (A) or 12 days (▲) (B) after inoculation, test substance (0.2 ml) was injected intravenously. Control: ×; MMC 0.4 mg/kg: □, 5 mg/kg: ◇, 10 mg/kg: ■; Suc(II)-MMC 0.4 mg eq. MMC/kg: △, 10 mg eq. MMC/kg: ▲; Lac-Suc-MMC 0.4 mg eq. MMC/kg: ○, 10 mg eq. MMC/kg: ●. (A-2), (B-2): Each point represents the mean ± S.D. ($n = 5$). *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$ vs. control.

Suc(II)-MMC (10 mg eq. MMC/kg) and Lac-Suc-MMC (10 mg eq. MMC/kg). A fair loss of body weight was observed in these groups (Fig. 4(A-2)). At 10 mg eq. MMC/kg, MMC was lethally toxic, but Suc(II)-MMC and Lac-Suc-MMC were not. The conjugates were considered to be less toxic than MMC. Fig. 4(B) shows the therapeutic efficacy of both conjugates when administered at 12 days post-inoculation. No markedly long survival was observed in any of the groups. Since lethal toxicity was not observed (Fig. 4(B-2)), each drug was considered not to work well against the tumor at the doses tested.

3.5. Distribution of free and conjugated MMCs in systemic circulation and liver after i.v. injection of the conjugates

The distribution of free and conjugated MMCs was examined after i.v. administration of Suc(II)-MMC and Lac-Suc-MMC at the dose of 4 mg eq. MMC/kg; this dose was chosen as intermediate dose between low and high doses used in the antitumor tests. Therefore, the result did not reflect exactly the drug distribution in the antitumor tests. However, it should give the biodistribution features of free and conjugated MMCs and

permit qualitative evaluation of the distribution of free and conjugated MMCs. The antitumor properties at the dose, 4 mg eq. MMC/kg, will be reported elsewhere. Table 3 indicates the plasma and liver concentration of free MMC and total (free and conjugated) MMC at 8 and 24 h after i.v. administration of Lac-Suc-MMC and Suc(II)-MMC. As to Lac-Suc-MMC, at both 3 and 12 days post-inoculation, MMC was observed in the liver mainly as a conjugated form, and free MMC was scarcely detected; though free MMC was detected at 8 h after injection at 12 days post-inoculation. On the other hand, for Suc(II)-MMC, MMC was hardly observed at all in the liver. At both 3 and 12 days post-inoculation, MMC was detected in plasma as a conjugated form at 8 h after administration. Neither free nor conjugated MMC was detected in the other time points.

4. Discussion

In liver metastasis induced by inoculation of M5076, amber flecks were spread out over the liver surface, and the liver became hypertrophic with progression of liver metastasis. In fact, at 12 days post-inoculation, the liver was approximately 1.5 times heavier than that of normal mice (data not shown). The liver surface, which is normally glossy, became rugged with the progression of liver metastasis, and the liver became fragile and crumbly in the advanced stage of metastasis. In normal animals, Lac-Suc was selectively distributed to the liver, and it was shown to be able to behave as a liver targeting drug carrier (Kato et al., 2001). However, it is possible that the biodistributions of Lac-Suc-FTC and Suc-FTC are influenced by the pathological state after M5076 inoculation. Therefore, we investigated the biodistributions of Lac-SucFTC and Suc-FTC in mice inoculated with M5076 as a liver metastasis tumor model. The experiments were performed at both the early and advanced stages of liver metastasis. There is a possibility that tissue weight, in particular the liver weight, may influence the evaluation of the distribution. Both the concentration and amount of Lac-Suc-FTC or Suc-FTC were checked at each stage of metastasis and the results were evaluated taking the above into account.

It is well known that FITC tagged substances have been found to be stable, non-reactive, non-toxic, little quenching and to behave similarly to the non-tagged parent substance in vivo (McDonagh and Williams, 1984; Gerlowski and Jain, 1986). In addition, it was also confirmed that the bond between Suc and fluorescein moiety was stable in the body (Kamiyama et al., 1999) and that Lac-Suc-FTC was little quenched in vitro

Table 3
Plasma or liver concentration of free and total MMCs at 8 and 24 h after i.v. administration of Lac-Suc-MMC and Suc(II)MMC at 4 mg eq·MMC/kg in mice

Conjugate			Concentration (μg/ml or g)[a]			
			3 day		12 day	
			8 h	24 h	8 h	24 h
Lac-Suc-MMC	Plasma	Free MMC	N.D.[b]	N.D.	N.D.	N.D.
		Total MMC	N.D.	N.D.	N.D.	0.06
	Liver	Free MMC	N.D.	N.D.	0.13 ± 0.08	N.D.
		Total MMC	0.39 ± 0.09	0.21 ± 0.03	0.14 ± 0.09	0.17 ± 0.31
Suc(II)-MMC	Plasma	Free MMC	N.D.	N.D.	N.D.	N.D.
		Total MMC	0.11 ± 0.16	N.D.	0.04 ± 0.05	N.D.
	Liver	Free MMC	N.D.	N.D.	N.D.	N.D.
		Total MMC	N.D.	N.D.	N.D.	N.D.

The detection limit for MMC in HPLC: 0.04 μg/ml.
[a] The results are expressed as the mean ± S.D. of four mice.
[b] N.D. means 'non-detected'.

(Kato et al., 2001). Therefore, the stability and the quenching effect of both fluorescent-carriers were considered to be negligible for in vivo quantitative analysis in this experiment. Moreover, biodistribution of Suc(II)-FTC was investigated in our earlier report (Kato et al., 2000c). That result indicated that further succinylation of *N*-succinylchitosan to 1.1 mol per sugar unit little influenced its biodisposition characteristics. Therefore, Suc-FTC used in this study reflects nearly the biodistribution of Suc(II).

As to the biodisposition of Lac-Suc-FTC, the liver concentration at 3 days post-inoculation at 1 h post-injection, being 11.3% of dose per g (Fig. 1(A-1)), was lower than that at 1 h post-injection in normal mice in which 22.8% of dose per g (Kato et al., 2001). The $AUC(C)^{liver}_{0-48\ h}$ value at 3 days post-inoculation was slightly lower than that of normal mice (Table 1). The hepatic uptake profile might be reduced somewhat by liver tumor metastasis. The total amount recovered from the tested tissues at 48 post-injection of Lac-Suc-FTC was much lower than the dose. Since urinary excretion was also very small, the localization of Lac-Suc-FTC to untested tissues was possibly high; experiments to test this speculation are in progress. The chitin or chitosan derivatives are supposed to undergo more degradation in tumor-bearing mice because of the elevated lysozymic activity in these animals (Klockars, 1974; Akema, 1974). However, the level of urinary excretion was very low in these metastatic mice (Fig. 3) and similar to or less than that in normal mice (Kato et al., 2001), which suggested that the biodegradation of Lac-Suc-FTC is hardly altered by metastasis.

The biodistribution of Suc-FTC at 3 days post-inoculation was similar to that in normal mice (Kamiyama et al., 1999; Kato et al., 2000a). Suc-FTC showed high-level and long-term retention in plasma (Fig. 2(A)). However, a small distribution of Suc-FTC was observed in the spleen, lung, liver and kidney. The distribution in the lung was greater initially and decreased from 8 h post-injection. The distribution to the liver and spleen tended to increase with time following i.v. injection. The majority of M5076 cells are distributed to the lung immediately after i.v. inoculation, and then are gradually distributed to other tissues including the liver, kidney and spleen (Hart et al., 1981). At the early stage of liver metastasis, i.e. 3 days post-inoculation, M5076 cells are distributed to the tissues mentioned above and are also capable of recirculation. Accordingly, it is conceivable that the phagocytosis of carriers, in this case Suc-FTC, by M5076 cells and the subsequent transfer of the cells may be partly responsible for the tissue distribution. Anyway, at 3 days post-inoculation, the targeting ability of Suc-FTC to the liver was minimal. On the other hand, at 12 days post-inoculation, Suc-FTC was gradually distributed to the liver and spleen and was eliminated from plasma relatively quickly (Fig. 2(B)). The $AUC^{plasma}_{0-48\ h}$ at 12 days post-inoculation was less than that at 3 days post-inoculation. The liver localization was increased at 12 days post-inoculation; especially, the localized amount was much raised. The increase in liver size was partly responsible, and further the EPR effects may be related to the increase in localization at the advanced stage. At 12 days after inoculation, the amount of Suc-FTC accumulated in the liver (32.4 ± 5.6 μg) was higher than that of Lac-Suc-FTC (20.3 ± 4.8 μg) at 48 h post-injection (Fig. 2(B-2)), although this difference was not significant ($P > 0.05$). It might be thought that Suc is available for liver targeting at the advanced stages of metastasis, i.e. in the deteriorated liver. The urinary excretion was small at 12 days as well as at 3 days post-inoculation, suggesting that the biodegradation or excretion of Suc is not promoted by metastasis.

One mouse died at 2 days post-administration when treated with Suc(II)-MMC at a dose of 10 mg eq. MMC/kg at 3 days post-inoculation (Fig. 4(A-1)). This rapid death was supposed to be due to the reason other than drug toxicity, because Suc(II)-MMC was not lethally toxic at 10 mg eq. MMC/kg according to the earlier study on antitumor effect against Sarcoma 180-tumor bearing mice (Kato et al., 2000b). As earlier reported (Sato et al., 1996), it was difficult to inject the high viscous conjugated drug solution safely into the vein probably because of prevention of blood flow. The tested samples, i.e. Suc(II)-MMC, were also too viscous especially at a dose of 10 mg eq.

MMC/kg. Further, the patterns of the loss of body weight were different between MMC and conjugates. Therefore, it was supposed that the sample viscosity should be responsible for the above rapid death of one mouse with the conjugate solution. The ILS values in MMC at 5 mg/kg, Suc(II)-MMC and Lac-Suc-MMC at 10 mg eq. MMC/kg are 62.5, 75.0 and 91.3 (%), respectively. The ILS values of Lac-Suc-MMC in M5076-bearing mice tended to be higher than those of Suc(II)-MMC at low and high doses, respectively (data not shown). However, one mouse showed a long survival, i.e. more than 50 days, when administered Suc(II)-MMC at a dose of 10 mg eq. MMC/kg. In addition, if ILS value in Suc(II)-MMC at 10 mg eq. MMC/kg was calculated using four mice except one that died at 5 days, the value is more than 110%. Although MMC at a dose of 5 mg/kg exhibited the similar survival when compared with the conjugates at a dose of 10 mg eq. MMC/kg, both conjugates extremely show the elongation of the lifespan by other schedule (unpublished data); this observation will be reported elsewhere. On the other hand, when administered at 12 days post-inoculation, no significant elongation of survival was observed with either conjugate (Fig. 4(B-1)). The administration of conjugates at 12 days post-inoculation appeared to be too late to cure the mice.

The distribution of MMC after i.v. injection of free MMC was not performed, because no MMC was detected at 8 h when injected to mice at 5 mg/kg (Kato et al., 2000b). The liver localization of Lac-Suc-MMC was superior to that of Suc(II)-MMC, which was consistent with the biodistribution profiles of their carriers; however, these values in Table 3 were lower than those calculated from the biodistribution study using FITC-labeled polymers. One explanation for these results may be that MMC, whether large or small, was degraded in acidic conditions or by enzyme (Beijnen and Underberg, 1985; Song et al., 1996). Table 3 also suggested that not only the concentration of MMC in the liver but also that in the systemic circulation is one of the major factors affecting the antitumor activity because Suc(II)-MMC, not detected in the liver at both tested time points, exhibited a good antitumor effect similar to Lac-Suc-MMC. Long systemic circulation of MMC might be available for efficacy on liver metastasis; long systemic retention of Suc(II)-MMC permitted the long retention of free MMC by the gradual release in the blood stream (Kato et al., 2000c). Further, Lac-Suc-MMC was considered to be targeted predominantly to the liver parenchymal cells. For Lac-Suc-MMC to be effective against a tumor, the drug targeted to the parenchymal cells must escape inactivation and diffuse to the diseased region. Although Lac-Suc-MMC was well targeted to the liver, it did not show antitumor effect surpassing that of Suc(II)-MMC, which might be related to instable properties of MMC in acidic or biological medium (Beijnen and Underberg, 1985; Song et al., 1996). In short, although Lac-Suc-MMC was taken up by liver cells, MMC released from Lac-Suc-MMC might be easily degraded in cells by acidic pH and metabolic enzymes before exhibiting action at the surrounding diseased part. Such inactivation is more serious for Lac-Suc-MMC localized into liver cells in comparison with Suc(II)-MMC residing long in the blood flow.

In conclusion, this study clarified the localization characteristics of Lac-Suc and Suc in the liver at the early and advanced stages of liver metastasis. Lac-Suc was concentrated more effectively in the early metastatic stage, while Suc was localized more to the liver in the advanced stage of liver metastasis. The conjugates of MMC with Lac-Suc and Suc functioned effectively in the early metastatic stage. Namely, in early metastatic stage; it was considered that both systemic long-retention and liver localization of MMC tended to be available for effectiveness against M5076 metastasis. Thus, both carriers were found potentialities as drug carriers for therapeutics of liver metastatic tumor in the early stage. The examination of therapeutic efficacy of Lac-Suc-MMC and Suc(II)-MMC with various schedules is now in progress.

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